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Combined Therapy of Dietary Fish Oil and Stearoyl-CoA Desaturase 1 Inhibition Prevents the Metabolic Syndrome and Atherosclerosis

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Background—Stearoyl-CoA desaturase 1 (SCD1) is a critical regulator of energy metabolism and inflammation. We have previously reported that inhibition of SCD1 in hyperlipidemic mice fed a saturated fatty acid (SFA)-enriched diet prevented development of the metabolic syndrome, yet surprisingly promoted severe atherosclerosis. In this study we tested whether dietary fish oil supplementation could prevent the accelerated atherosclerosis caused by SCD1 inhibition.

Methods and Results—LDLr^{-/-}, ApoB^{100/100} mice were fed diets enriched in saturated fat or fish oil in conjunction with antisense oligonucleotide (ASO) treatment to inhibit SCD1. As previously reported, in SFA-fed mice, SCD1 inhibition dramatically protected against development of the metabolic syndrome, yet promoted atherosclerosis. In contrast, in mice fed fish oil, SCD1 inhibition did not result in augmented macrophage inflammatory response or severe atherosclerosis. In fact, the combined therapy of dietary fish oil and SCD1 ASO treatment effectively prevented both the metabolic syndrome and atherosclerosis.

Conclusions—SCD1 ASO treatment in conjunction with dietary fish oil supplementation is an effective combination therapy to comprehensively combat the metabolic syndrome and atherosclerosis in mice. (*Arterioscler Thromb Vasc Biol.* 2010;30:24-30.)

Key Words: saturated fat ■ fish oil ■ atherosclerosis ■ inflammation ■ metabolic syndrome

It has been estimated that nearly a quarter of American adults have the metabolic syndrome.¹⁻³ Inhibition of stearoyl-CoA desaturase 1 (SCD1) has been proposed as an attractive strategy for preventing most aspects of the metabolic syndrome including obesity,⁴⁻⁹ insulin resistance,^{4,6,10,11} hypertriglyceridemia,^{6,12-14} and hepatic steatosis.^{8,9,12,15,16} However, several unwanted side effects are associated with SCD1 inhibition or deletion, including alopecia¹⁷⁻²⁰ and accelerated atherosclerosis.^{21,22} We have previously shown that the accelerated atherosclerosis seen with SCD1 ASO-mediated inhibition is associated with saturated fatty acid (SFA) enrichment of macrophage membranes, and enhanced proinflammatory signaling through toll-like receptor 4 (TLR4).²¹ Likely through a similar mechanism, mice lacking SCD1 have enhanced dextran sulfate sodium (DSS)- and bacterial-induced inflammatory gene expression and exaggerated colitis.²³ These recent studies²¹⁻²³ suggest that SCD1 may serve a protective function against proinflammatory signaling.

It is reasonable to assume that many of the inflammation-linked side effects seen with SCD1 inhibition stem from the abnormal accumulation of SCD1 substrates, saturated fatty acids, in multiple tissues. Indeed, there is a large body of evidence that SFAs are potent proinflammatory molecules, linking these SCD1 substrates to a number of inflammatory diseases.²⁴⁻³⁴ In fact, recent evidence suggests that SFAs can activate multiple toll-like receptors (TLRs), which play a key role in innate immunity.^{24,28,29,31-34} Furthermore, TLR4 is necessary for SFAs to induce obesity, insulin resistance, and vascular inflammation in rodents.²⁴⁻²⁷ Therefore, one of the key roles of SCD1 may be to suppress inflammation by preventing excessive accumulation of SFA-derived TLR4 ligands. Interestingly, long chain ω -3 polyunsaturated fatty acids (ω -3 PUFA) have been shown to counteract SFA-induced TLR4 activation in cultured macrophage and dendritic cell systems.^{28,30-33} In parallel, there is strong evidence that dietary ω -3 PUFA supplementation can effectively blunt inflammation and related diseases in vivo.³⁴⁻³⁶ Therefore, we

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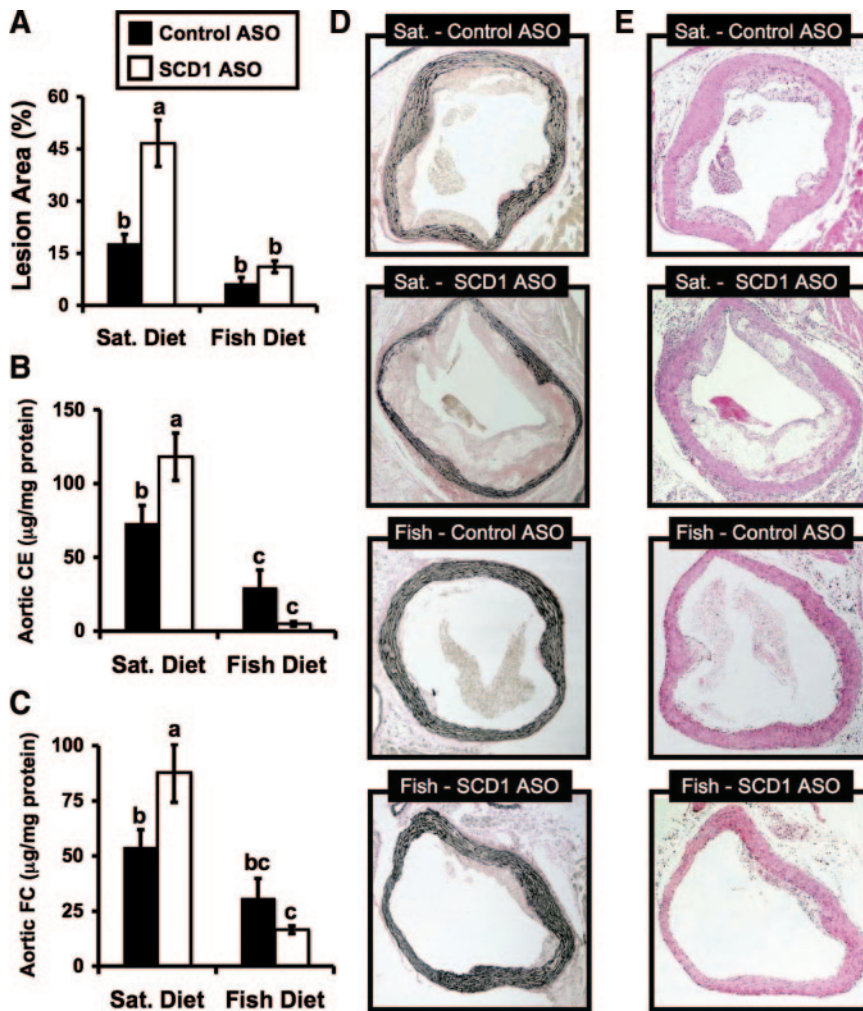


Figure 1. Dietary fish oil supplementation prevents SCD1 ASO-driven atherosclerosis in $LDLr^{-/-}$, $ApoB^{100/100}$ mice. Starting at 6 weeks of age, male mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω -3 fatty acids (Fish) for 20 weeks in conjunction with biweekly injections (25 mg/kg) of a non-targeting control ASO ■ or SCD1 ASO □. A, En face morphometric analysis of total aortic lesion area. Data shown in panel A represent the mean \pm SEM from 6 mice per group. GLC analysis of aortic cholesteryl ester (B) and free cholesterol (C) was determined. Data in panels B and C represents the mean \pm SEM from 8 to 15 mice per group. Values not sharing a common superscript differ significantly ($P < 0.05$). D, Representative Verhoeff-van Gieson stained sections of proximal aortae from mice treated with diet and ASO for 20 weeks. E, Representative hematoxylin and eosin-stained sections of proximal aortae from mice treated with diet and ASO for 20 weeks.

reasoned that dietary supplementation with fish oil-derived ω -3 PUFAs may prevent the SFA-driven TLR4 hypersensitivity and accelerated atherosclerosis seen with SCD1 inhibition.²¹

Methods

Male low-density lipoprotein receptor-deficient ($LDLr^{-/-}$), apolipoprotein B 100 only ($ApoB^{100/100}$) mice were treated with antisense oligonucleotides (ASOs) to inhibit SCD1 while consuming diets containing 0.1% (w/w) cholesterol and 12% of energy as primarily either SFA-enriched fat (palm oil) or long chain ω -3 PUFA-enriched fat (fish oil) for 20 weeks as previously described.²¹ Detailed descriptions of diets, materials, and experimental methods are available in the supplemental materials (available online at <http://atvb.ahajournals.org>).

Results

SCD1 ASO Treatment and Dietary Fish Oil Reduce SCD1 Expression in a Tissue-Specific Manner, Resulting in Protection Against Diet-Induced Obesity and Insulin Resistance

There is a large body of evidence that either SCD1 inhibition or dietary fish oil alone can protect against diet induced obesity and insulin resistance in rodents.^{4–11,30–36} However,

the possibility that the combination of these 2 treatments can act synergistically to improve the metabolic syndrome has never been addressed. Interestingly, both SCD1 ASO treatment and dietary fish oil can diminish SCD1 expression in a tissue-specific manner (supplemental Figure 1), resulting in dramatic protection against diet-induced obesity and insulin resistance (supplemental Figure 2). A detailed description of these anticipated metabolic improvements is provided in the supplemental materials.

Dietary Fish Oil Supplementation Prevents SCD1 ASO-Driven Atherosclerosis

We have previously shown that the SCD1 inhibition in hyperlipidemic mice fed either SFA-rich or monounsaturated fatty acid (MUFA)-rich diets results in more extensive atherosclerosis.²¹ This unforeseen side effect of SCD1 inhibition was associated with SFA enrichment of plasma lipids and macrophage membranes, together with enhanced TLR4-driven proinflammatory signaling. Importantly, long chain ω -3 PUFAs have been shown to prevent SFA-induced TLR4 activation in cultured cells.^{28,31–34} Therefore, we set out to test whether this relationship held up in vivo by examining atherosclerosis, a complex disease with an inflammatory component that is promoted by both

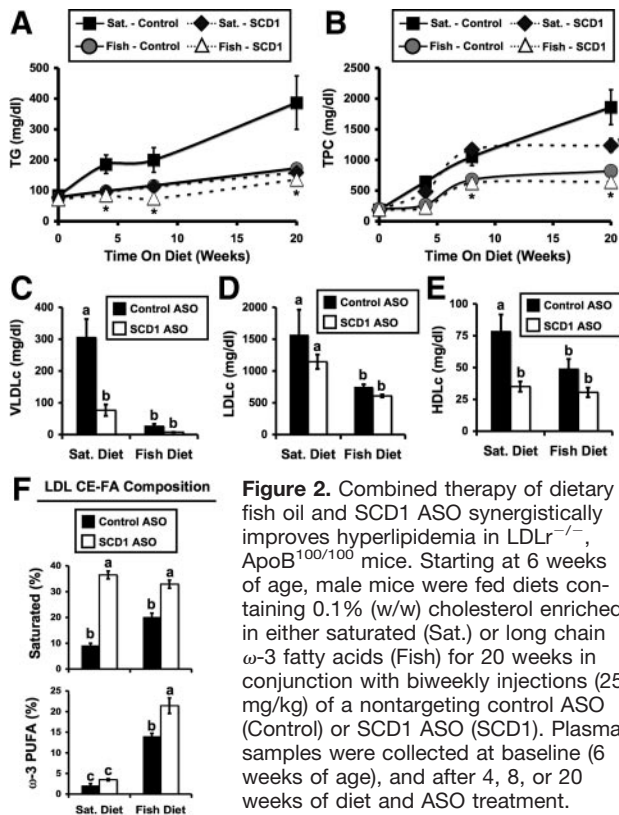


Figure 2. Combined therapy of dietary fish oil and SCD1 ASO synergistically improves hyperlipidemia in $LDL^{-/-}$, ApoB^{100/100} mice. Starting at 6 weeks of age, male mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω -3 fatty acids (Fish) for 20 weeks in conjunction with biweekly injections (25 mg/kg) of a nontargeting control ASO (Control) or SCD1 ASO (SCD1). Plasma samples were collected at baseline (6 weeks of age), and after 4, 8, or 20 weeks of diet and ASO treatment.

Plasma triglycerides (A) and total

plasma cholesterol (TPC; B) were measured enzymatically. Data shown in panels A and B represents the mean \pm SEM from 5 to 8 mice per group, *significantly different than the saturated diet fed control ASO treated group, within each time point ($P < 0.05$). Panels C through E represent cholesterol levels in very low-density lipoproteins (VLDLc), low-density lipoproteins (LDLc), and high-density lipoproteins (HDLc) in mice receiving dietary and ASO treatment for 20 weeks. Data shown in panels C through E represent the mean \pm SEM from 6 mice per group, and values not sharing a common superscript differ significantly ($P < 0.05$). F, Fatty acid (FA) composition (% of total FA as saturated or long chain ω -3 [eicosapentaenoic and docosahexaenoic] fatty acids) of LDL cholesteryl esters (LDL CE-FA). Data shown in panel F represent the mean \pm SEM ($n = 5$ per group), and values not sharing a common superscript differ significantly ($P < 0.05$).

SFA^{37,38} and TLR4 activation.^{39,40} As previously demonstrated, en face morphometric analysis showed that SCD1 ASO treatment in mice fed a SFA-rich diet resulted in a 2.7-fold increase in total aortic lesion area, compared to mice treated with a control ASO (Figure 1A). In parallel, SCD1 inhibition in mice fed a SFA-rich diet also resulted in increased concentrations of aortic cholesteryl ester (Figure 1B), aortic free cholesterol (Figure 1C), and abundant areas of necrosis with visible cholesterol crystals (Figure 1D), all indicating accelerated atherosclerosis. In contrast, in mice fed a fish oil diet, SCD1 ASO treatment had no significant effects on lesion area (Figure 1A), aortic cholesteryl ester (Figure 1B), and aortic free cholesterol (Figure 1C), or lesion complication (Figure 1D and 1E), compared to control ASO treated mice. The chemical measurement may have been the most precise quantification of atherosclerosis, and mice fed a fish oil diet had significantly less aortic cholesteryl ester, compared to

SFA-fed mice regardless of ASO treatment (Figure 1B). After 20 weeks of induction, the rank order of aortic cholesteryl ester for the groups was: SFA-fed/SCD1 ASO (118 μ g/mg protein) > SFA-fed/Control ASO (73 μ g/mg protein) > Fish-oil fed/Control ASO (29 μ g/mg protein) > Fish oil-fed/SCD1 ASO (5 μ g/mg protein).

Dietary Fish Oil Supplementation and SCD1 ASO Treatment Improve Atherogenic Hyperlipidemia in a Complimentary Fashion

In agreement with previous reports,^{6,12–14} SCD1 inhibition alone and dietary fish oil alone prevented diet-induced hypertriglyceridemia, which was apparent after only 4 weeks of either treatment (Figure 2A). After 8 weeks of treatment, the rank order of the groups for plasma TG was: SFA-fed/Control ASO (201 mg/dL) > SFA-fed/SCD1 ASO (118 mg/dL) > Fish oil-fed/Control ASO (113 mg/dL) > Fish oil-fed/SCD1 ASO (76 mg/dL). In contrast to plasma TG, SCD1 ASO treatment only modestly reduced total plasma cholesterol (TPC) after 20 weeks in mice fed the SFA diet. TPC was not significantly altered after 4 or 8 weeks of this treatment (Figure 2B). Furthermore, in the fish oil-fed group, SCD1 ASO treatment did not produce a TPC lowering effect (Figure 2B). When lipoprotein cholesterol distribution was analyzed, we discovered that SCD1 inhibition alone and fish oil feeding alone decreased VLDL cholesterol compared to their respective controls (Figure 2C), but the 2 treatments together were not synergistic in lowering VLDLc. Interestingly, SCD1 ASO treatment had no effect on LDLc regardless of diet (Figure 2D). In contrast, dietary fish oil significantly reduced LDLc, compared to SFA-fed groups, regardless of ASO treatment (Figure 2D). Furthermore, both SCD1 ASO treatment and dietary fish oil caused significant reductions in plasma HDLc (Figure 2E). As previously described,²¹ SCD1 ASO treatment caused marked enrichment of LDL cholesteryl esters (LDL-CE) with saturated FA in both diet groups (Figure 2F). Also, dietary fish oil supplementation resulted in the expected ω -3 PUFA-enrichment in LDL-CE (Figure 2F). Interestingly, ASO-mediated inhibition of SCD1 in fish oil-fed mice caused significantly more ω -3 PUFAs to be incorporated in LDL-CE, compared to control ASO treated mice. It seems the primary effects of SCD1 inhibition alone are: diminished plasma triglyceride, decreased VLDLc, and enrichment of plasma lipoproteins with SFA. In contrast, dietary fish oil effectively reduces both plasma TG and LDLc levels. Collectively, the effects of SCD1 ASO and dietary fish oil synergistically improve atherogenic hyperlipidemia, likely through independent mechanisms.

Dietary Fish Oil Supplementation Prevents SCD1 ASO-Driven TLR4 Hypersensitivity in Macrophages

SCD1 ASO treatment for 6 weeks reduced the palmitoleate (16:1) to palmitate (16:0) ratio in macrophage PL in both diet groups (Figure 3A). This was anticipated because we have previously demonstrated that SCD1 ASO treatment reduces macrophage SCD1 expression.²¹ Dietary fish oil

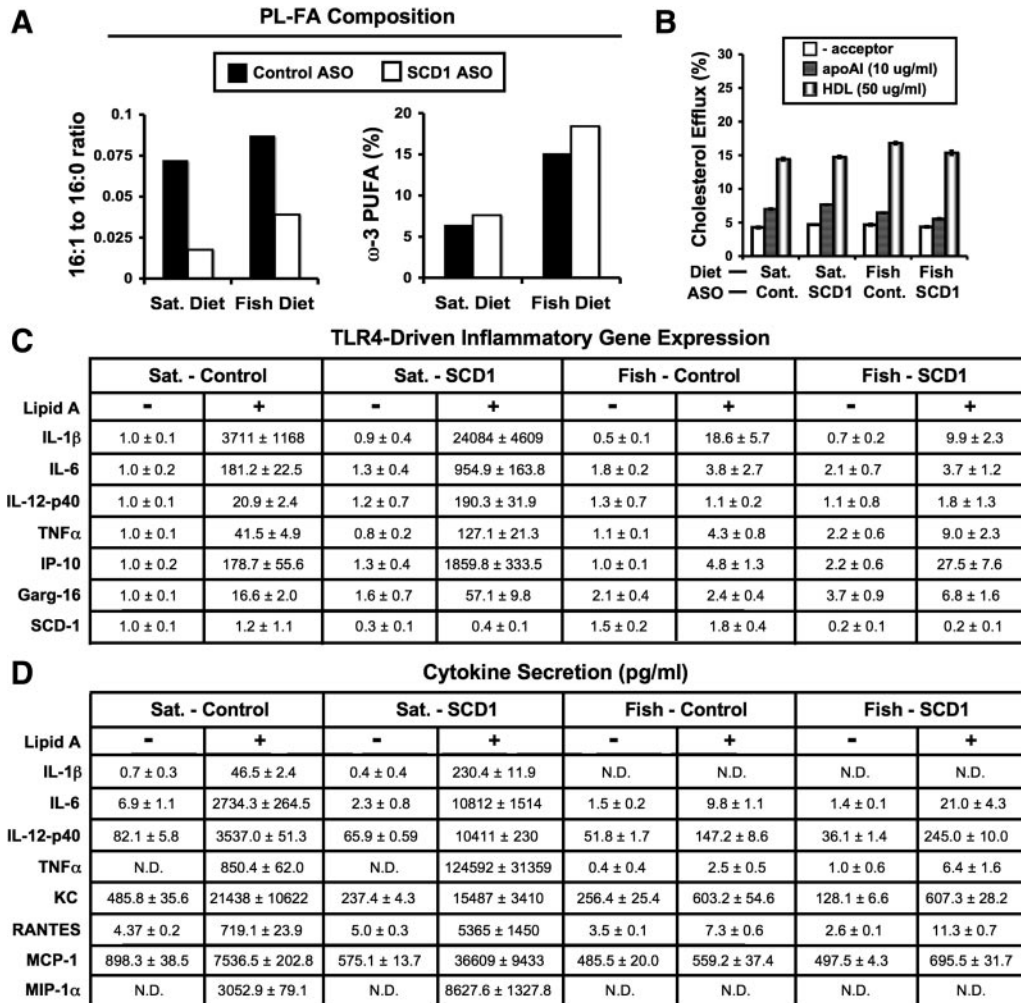


Figure 3. Dietary fish oil supplementation prevents SCD1 ASO-driven TLR4 hypersensitivity in macrophages. Starting at 6 weeks of age, male mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω -3 fatty acids (Fish) for 6 weeks in conjunction with biweekly injections (25 mg/kg) of a nontargeting control ASO (Cont.) or SCD1 ASO (SCD1). After 6 weeks of diet and ASO treatment, freshly isolated thioglycollate-elicited macrophages were pooled ($n=5$ to 7 mice per pool) and cultured as described in materials and methods. A, Phospholipid fatty acid (PL-FA) composition (16:1 to 16:0 ratio and % of total FA as long chain ω -3 [eicosapentaenoic and docosahexaenoic] fatty acids) of freshly isolated (2-hour culture) macrophages. B, Macrophage cholesterol efflux to apoAI (10 μ g/mL) or high-density lipoprotein (HDL, 50 μ g/mL). C, TLR4-driven gene expression: Freshly isolated macrophages were treated with vehicle or 10 ng/mL Kdo₂-Lipid A (TLR4 agonist) for 6 hours, and mRNA levels were measured for interleukins 1 beta (IL-1 β), 6 (IL-6), and 12p40 (IL-12p40), tumor necrosis factor α (TNF α), C-X-C motif ligand 10 (IP-10), and glucocorticoid attenuated response gene 16 (Garg-16), and normalized to GAPDH. Data shown in panel C are expressed as the mean relative mRNA expression, where all values were normalized to the levels in the Sat. - Control vehicle treated group, and the SEM was calculated from triplicate plates for each pool. D, TLR4-driven cytokine secretion: Freshly isolated macrophages were treated with vehicle or 10 ng/mL Kdo₂-Lipid A (TLR4 agonist) for 6 hours, and cytokine levels (pg/mL) were measured for IL-1 β , IL-6, IL-12p40, TNF α , Chemokine C-X-C motif ligand 1 (KC), Regulated on Activation Normal T-cell Expressed and Secreted (RANTES), monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 1 alpha (MIP-1 α) using conditioned media. Data shown in panel D the mean \pm SEM from triplicate plates for each pool. N.D. indicates values not detectable.

alone did not alter the 16:1 to 16:0 ratio in macrophage PL, compared to PL isolated from SFA-fed mice (Figure 3A), and did not alter macrophage SCD1 mRNA levels (Figure 3C). However, dietary fish oil supplementation resulted in expected ω -3 PUFA-enrichment in macrophage PL (Figure 3A). As seen in plasma (Figure 2F) and the liver (supplemental Figure 3D), ASO-mediated inhibition of SCD1 in mice fed a fish oil diet caused a trend toward increased ω -3 PUFA incorporation into macrophage PL, compared to control ASO-treated mice. It has previously been demonstrated that macrophage SCD1 plays a role in cellular

cholesterol efflux,⁴¹ which could subsequently impact atherogenesis. However, we did not see any appreciable effects of diet or SCD1 ASO treatment on cellular cholesterol efflux to either lipid-free apoAI or HDL (Figure 3B). More importantly, when macrophages isolated from SCD1 ASO-treated mice fed a SFA-rich diet were challenged with a specific TLR4 agonist (10 ng/mL Kdo₂-Lipid A), marked hypersensitivity was apparent, both at the mRNA level (Figure 3C) and the level of protein secretion (Figure 3D) as previously described.²¹ In support of this, TLR4-dependent upregulation of inflammatory gene expression

was much more robust in macrophages isolated from SFA-rich diet-fed mice treated with SCD1 ASO, compared to macrophages isolated from their counterparts treated with control ASO (Figure 3C). Likewise, TLR4-dependent promotion of inflammatory cytokine secretion was much more robust in macrophages isolated from SFA-rich diet-fed SCD1 ASO-treated mice, compared to macrophages isolated from their counterparts treated with control ASO (Figure 3D). In contrast, in mice fed a ω -3 PUFA-rich diet, SCD1 ASO treatment did not result in TLR4 hypersensitivity (Figure 3C and 3D). Interestingly, plasma levels of inflammatory cytokines were relative low in all the experimental groups (supplemental Table II) and were not significantly different with SCD1 ASO treatment. Collectively, these data suggest that SCD1 ASO-driven TLR4 hypersensitivity in macrophages can be prevented by dietary fish oil supplementation.

Combined Therapy of Dietary Fish Oil and SCD1 ASO Prevents Diet-Induced Hepatic Steatosis

Mice lacking SCD1 are protected against diet- and genetically-induced hepatic steatosis.^{13,14,17,20,21} Likewise, dietary ω -3 PUFA supplementation protects against hepatic steatosis in a number of experimental models.^{6,22} Interestingly, the dual therapy of dietary fish oil and SCD1 ASO treatment resulted in near complete prevention of diet-induced hepatic steatosis (supplemental Figure 3). A detailed description of these data are provided in the supplemental material.

Discussion

The search for metabolic syndrome targets has strongly supported SCD1 inhibitors as an attractive option for preventing obesity, insulin resistance, hypertriglyceridemia, and hepatic steatosis.^{4–16} Unfortunately, there are severe side effects associated with diminished SCD1 activity in mice, including skin pathology^{17–20} and accelerated atherosclerosis.^{21,22} These warning signs have unfortunately impeded many SCD1 inhibitor programs,⁴² without complete understanding of the etiology of these complex side effects. It is logical to assume many of the side effects seen with SCD1 inhibition stem from the abnormal accumulation of SFAs in multiple tissues. Indeed, SFAs are potent proinflammatory molecules^{24–34} and have been linked to innate immunity.^{24,28,29,31–34} Therefore, SCD1 may indirectly suppress inflammation by preventing SFA-induced activation of TLR4. The concept of fatty acids regulating inflammation is not unique to SFAs. In fact, long chain ω -3 PUFA from fish oil have been shown to inhibit inflammation, and more importantly, counteract SFA-induced TLR4 activation in cultured cells.^{28,30–33} This study provides new evidence that this reciprocal relationship between SFA and ω -3 PUFA in modulating inflammation also holds true in vivo, and can be exploited to protect against multiple metabolic diseases.

The question now becomes: how does the combination of SCD1 ASO treatment and dietary fish oil synergize to

comprehensively prevent the development of obesity, insulin resistance, hyperlipidemia, hepatic steatosis, and atherosclerosis? There are likely both shared and independent mechanisms by which these treatments mediate their effects. When given as a monotherapy, SCD1 ASO treatment results in striking protection against diet-induced obesity,^{7,21} insulin resistance,^{7,10,21} hypertriglyceridemia,^{7,21} and hepatic steatosis.^{7,21} Unfortunately, SCD1 ASO treatment promotes severe atherosclerosis in hyperlipidemic mice fed either a SFA- or MUFA-rich diet.²¹ We believe that dietary fish oil supplementation is able to prevent SCD1 ASO-driven atherosclerosis through at least 3 independent mechanisms: (1) lowering LDLc, (2) enriching the remaining LDL-CE in atheroprotective ω -3 PUFAs, and (3) counteracting SFA-driven inflammation. In support of this, dietary fish oil lowered LDLc by 47% to 53%, compared to SFA-fed mice regardless of ASO treatment (Figure 2D). However, the LDL-CE remaining in fish oil fed mice treated with SCD1 ASO was highly enriched in ω -3 PUFAs, yet enriched in SFA to the same extent as SFA-fed SCD1 ASO-treated mice (Figure 2F). These results indicate that dietary fish oil does not diminish SCD1 ASO-mediated SFA enrichment of plasma lipids, but rather shifts the fatty acid composition to be more polyunsaturated, which has the potential to diminish the production of VLDLc⁴⁵ (Figure 2C). In this regard, it has been previously demonstrated that long chain fatty acids such as docosahexaenoic acid (DHA) can inhibit VLDL secretion by promoting post-ER presecretory proteolysis (PERPP)-mediated degradation of apolipoprotein B⁴⁶ or endoplasmic reticulum (ER) stress-related degradation of apoB.⁴⁷ Although we did not directly measure oxidant stress or PERPP, we saw no indication that either fish oil or SCD1 ASO promoted hepatic ER stress (supplemental Figure 4).

It is important to note that SCD1 ASO treatment unexpectedly results in a dramatic HDLc reduction in hyperlipidemic mice fed a SFA-rich diet.²¹ Based on this, it has recently been speculated that SCD1 ASO-driven HDLc lowering may be the cause of accelerated atherosclerosis seen under these conditions.²² However, our data suggest that HDLc lowering plays little, if any, role in SCD1 ASO-driven accelerated atherosclerosis. In support of this, dietary fish oil alone actually decreased HDLc, compared to SFA-fed mice (Figure 2E), yet atherosclerosis was also decreased in fish oil-fed mice. Most importantly, fish oil supplementation did not prevent SCD1 ASO-mediated reductions in HDLc seen in SFA-fed mice (Figure 2E). In fact, the mice treated with SCD1 ASO and fed dietary fish oil had the lowest HDLc of any group with the rank order of the 4 groups being: SFA-fed/Control ASO (79 mg/dL) > Fish oil-fed/Control ASO (49 mg/dL) > SFA-fed/SCD1 ASO (35 mg/dL) > Fish oil-fed/SCD1 ASO (30 mg/dL). Collectively, these data suggest that HDLc modulation is not the primary mechanism by which dietary fish oil protects against SCD1 ASO-driven atherosclerosis.

In addition to reducing plasma lipoprotein levels, dietary fish oil prevents SCD1 ASO-driven TLR4 hypersensitivity (Figure 3C). This may be attributable, in part, to the

enrichment of macrophage membranes with long chain ω -3 PUFAs (Figure 3A), which are known to prevent SFA-driven TLR4 activation.^{28,30–33} Importantly, SCD1 ASO treatment results in marked accumulation of SFA in plasma, multiple tissues, and isolated macrophages (Figure 2F, supplemental Figure IIID, and Figure 3A,²¹). However, this SFA enrichment is not prevented by dietary fish oil supplementation (Figure 2F, supplemental Figure IIID, and Figure 3A). Hence, even in the face of massive SCD1 ASO-driven SFA accumulation, moderate dietary ω -3 PUFA supplementation can prevent SFA-driven inflammation (Figure 3C) and atherosclerosis (Figure 1). This makes it tempting to speculate that the other diverse pathologies associated with genetic deletion of SCD1,^{11,17–20,23} including alopecia, might likewise be ameliorated by the anti-inflammatory effects of dietary fish oil. Interestingly, during the preparation of this manuscript, a recent study warned that previous work^{29–33} describing SFA-mediated activation of TLR4 or TLR2 may have been confounded by contamination of the fatty acid vehicle (BSA) with LPS or bacterial lipoproteins.⁴⁸ Importantly, because in vivo dietary feeding of long-chain fatty acids does not require a BSA vehicle, this is likely not the only explanation for SFA-induced TLR4 activation. Rather, our results provide in vivo evidence that saturated fatty acids do indeed promote TLR4-dependent signaling and that n-3 PUFAs can antagonize SFA-driven TLR4 hypersensitivity (Figure 3). However, whether fatty acids exert their effects through direct TLR4 agonism or by modulating membrane organization is still a matter of debate and requires further work.

It is important to point out that ASO-mediated inhibition of SCD1 does not alter SCD1 protein expression in the skin or result in alopecia.²¹ This tissue-specific pattern of inhibition seen with in vivo ASO administration has been documented previously^{43,44} and is ideal for SCD1 inhibition where tissue specificity is required to avoid unwanted side effects. In a recent study by MacDonald and colleagues,²² it was speculated that the accelerated atherosclerosis seen with genetic SCD1 deficiency was in part attributable to dermal inflammation.²² This is unlikely to be the primary mechanism for the accelerated atherosclerosis, because SCD1 ASO treatment also results in severe atherosclerosis, without affecting skin SCD1 expression or alopecia.²¹ Therefore, ASO-mediated inhibition may provide a unique tissue-specific therapeutic strategy to avoid the skin pathology^{17–20} that would likely accompany small molecule SCD1 inhibitors without tissue specificity.

In summary, we have demonstrated that SCD1 ASO treatment protects against development of the metabolic syndrome but unfortunately promotes atherosclerosis in mice fed diets enriched in either SFA or MUFA.⁶ However, SCD1 ASO-driven atherosclerosis can be completely prevented by dietary fish oil (Figure 1). Importantly, the proinflammatory effects of SCD1 ASO treatment can be overcome by dietary ω -3 PUFA supplementation, and the dual therapy provides dramatic protection against atherogenic hyperlipidemia. Therefore, this synergistic dual therapy may provide a novel therapeutic approach for the metabolic syndrome and atherosclerosis.

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Disclosures

None.

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Supplemental Materials and Methods

Mice.

Male low density lipoprotein receptor deficient (LDLr^{-/-}), apolipoprotein B 100 only (ApoB^{100/100}) mice were used in this study. These mice were chosen based on previous reports documenting their “human-like” lipoprotein profile¹, atherosclerosis susceptibility^{1,2}, and responsiveness to dietary fatty acids². All mice were on a mixed background (~75% C57BL/6 and ~25% 129Sv/Jae). To inhibit SCD1, antisense oligonucleotides (ASOs) were given as previously described³, with minor modifications. Briefly, at 6 weeks of age, mice were switched from rodent chow to one of two synthetic diets containing 12% of energy as primarily either SFA-enriched fat (palm oil) or long chain ω-3 PUFA-enriched fat (fish oil), with 0.1% (w/w) cholesterol added. Please refer to Supplemental Table 1 for quality control analysis of dietary fatty acid composition. We have previously published the effects of SCD1 inhibition in SFA-fed mice³, and these data are included here strictly for comparative purposes. All experimental animals were sacrificed after 20 weeks of parallel dietary and ASO treatment, except for macrophage experiments where mice were sacrificed after 6 weeks of treatment. Mice were maintained in an American Association for Accreditation of Laboratory Animal Care-approved pathogen-free animal facility, and all experimental protocols were approved by the institutional animal care and use committee at the Wake Forest University School of Medicine.

Plasma Lipid and Lipoprotein Analyses. Plasma samples were collected by submandibular vein puncture at baseline (6 weeks of age chow fed animals), and after 4, 8, or 20 weeks of diet and ASO treatment. Detailed description of plasma lipid analyses has been previously described.³

Glucose homeostasis. Intraperitoneal glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed as previously described³ in mice that had been treated with diet and ASO for 16 weeks.

Quantification of atherosclerosis. *En face* morphometric and biochemical quantification of aortic atherosclerosis was conducted as previously described³ in mice that had been treated with diet and ASO for 20 weeks.

Hepatic lipid mass and fatty acid composition. Extraction of liver lipids and biochemical analyses were performed as previously described³ in mice that had been treated with diet and ASO for 20 weeks.

Hepatic ER stress. Total liver homogenates were prepared as previously described³, and ER stress was measured by immunoblotting using antibodies recognizing CHOP (Cell Signaling Technologies # 2895), protein disulfide isomerase (PDI, Cell Signaling Technologies # 2446), and BIP (Cell Signaling Technologies # 3177).

Peritoneal macrophage isolation and culture. For macrophage studies, mice received diet and ASO treatment for six weeks. Thereafter, thioglycolate-elicited peritoneal macrophages were pooled from five mice per group, and were cultured in 35-mm dishes as previously described.³ For TLR4 agonist studies, following two hours of culture, adherent macrophage were washed three times with PBS, and subsequently maintained in serum free RPMI-1640 for an additional two hours to dampen basal serum-induced signaling. Thereafter, cells were treated with vehicle (PBS) or 10 ng/ml of the defined TLR 4 agonist⁴ Kdo₂-Lipid A for six hours to examine inflammatory gene expression by qPCR as previously described³, and cytokine secretion was measured using a Bio-Plex mouse cytokine kit (Bio-Rad) using fluorescently labeled microsphere beads and a Bio-Plex suspension array system (Bio-Rad) according the to the manufacturer's instructions. For cholesterol efflux, freshly isolated macrophages were incubated with ³H-cholesterol for 8 hours. Thereafter, cells were gently washed four times with PBS, and then supplemented with 1 ml of efflux medium (serum-free RPMI-1640 and 0.1% fatty-acid-free BSA) in the absence or presence of cholesterol acceptors (10 µg/ml apoA-I or 50 µg/ml HDL) for 6 h at 37°C. To determine cholesterol efflux, the conditioned media were collected and centrifuged for 5 min at 16000 g to pellet cellular debris. An aliquot was removed and the radioactivity present in the incubation medium was determined by liquid-scintillation counting. The percentage of radiolabelled cholesterol released (% efflux) was calculated as: (dpm in medium after 6h / dpm in cells at zero time) x 100. For isolated macrophage experiments, the data shown in Figure 3 are representative of one pool of macrophages (n=5 mice per group) split into triplicate plates, and similar results were seen in an independent pool of macrophages (n=5-7 mice per group).

SCD Activity Assay. Hepatic SCD1 activity was measured in liver microsomes as previously described³, in mice that had been treated with diet and ASO for 20 weeks.

Quantitative Real-Time PCR (qPCR). RNA extraction and qPCR was conducted as previously described³ on pooled samples (n=5 per group). Messenger RNA levels for each gene represent the amount relative to the amount in the control ASO treated SFA diet-fed group, which was arbitrarily standardized to 100%. Primers used for qPCR have been previously described.³

Statistical Analysis. Data are expressed as the mean \pm standard error of the mean (SEM). All data were analyzed using two-way analysis of variance (ANOVA) using diet and ASO treatment as individual variables, followed by Student's t tests for post hoc analysis. Differences were considered significant at $p < 0.05$. All analyses were performed using JMP version 5.0.12 (SAS Institute; Cary, NC) software.

Supplemental Results

Dietary fish oil and SCD1 ASO treatment reduce SCD1 expression in a tissue-specific manner. To specifically inhibit SCD1 activity, we utilized antisense oligonucleotide (ASO) treatment as previously described³ in low density lipoprotein receptor knockout (LDLr^{-/-}), apolipoprotein B only (ApoB^{100/100}) mice, a mouse model of LDL-driven atherosclerosis. Mice were fed either a SFA-rich or a ω -3 PUFA-rich diet for 20 weeks. Regardless of diet, SCD1 mRNA levels were reduced by 99% in the liver and 78-93% in adipose tissue in mice treated with SCD1 ASO (Supplemental Figure 1, A and B). In agreement, hepatic SCD1 activity was reduced by > 95% after 20 weeks of SCD1 ASO treatment in mice fed the SFA-rich diet (Supplemental Figure 1C). As has been previously described^{5,6}, when compared to SFA-feeding, dietary ω -3 PUFA supplementation resulted in a 75% reduction in hepatic SCD1 mRNA levels in control ASO treated mice (Supplemental Figure 1A). This dietary fish oil-driven reduction of SCD1 mRNA occurred only in the liver, and not in adipose tissue (Supplemental Figure 1B) or macrophages (Figure 3C), indicative of tissue-specific regulation.

Combination therapy of dietary fish oil and SCD1 ASO prevents diet-induced obesity and insulin resistance. We previously demonstrated that ASO-mediated inhibition of SCD1 prevented diet-induced obesity and insulin resistance in LDLr^{-/-}, ApoB^{100/100} mice fed a SFA-rich diet.³ Likewise, it has been demonstrated that dietary fish oil supplementation can similarly improve obesity and insulin resistance.^{7,8} Therefore, we set out to determine if the combination therapy of dietary fish oil and

SCD1 ASO could act synergistically to further ameliorate diet-induced obesity and insulin resistance. In agreement with previous reports³, SCD1 inhibition prevented diet-induced obesity in LDLr^{-/-}, ApoB^{100/100} mice fed either diet (Supplemental Figure 2, A and B). Following 20 weeks of treatment, epididymal fat pad mass was reduced by ~85% in mice treated with SCD1 ASO fed the SFA-rich diet, compared to control ASO treated mice (Supplemental Figure 2B). In agreement with previous reports^{7,8}, dietary fish oil supplementation in control ASO-treated mice significantly reduced body weight gain, compared to control ASO-treated mice fed a SFA-rich diet (Supplemental Figure 2, A and B). It is important to point out that the effects of dietary fish oil and SCD1 ASO were not synergistic in reducing body weight. In addition to dramatic effects of adiposity, SCD1 ASO treatment significantly improved glucose tolerance in mice fed either diet (Supplemental Figure 2C). In control ASO treated mice, dietary fish oil modestly improved glucose tolerance, compared to SFA-feeding (Supplemental Figure 2C), regardless of ASO treatment. Furthermore, during insulin tolerance tests (ITT), SCD1 ASO treated mice had enhanced blood glucose disposal, compared to control ASO treated mice on either diet, indicating enhanced insulin sensitivity (Supplemental Figure 2D). However, the effects of dietary fish oil and SCD1 ASO were not synergistic in improving glucose and insulin tolerance. Collectively, these data support the notion⁹⁻²¹ that SCD1 ASO treatment alone is extremely effective in the prevention of diet-induced obesity and insulin resistance, and that SCD1 inhibition in the presence of dietary fish oil does not provide synergistic protection.

Combined therapy of dietary fish oil and SCD1 ASO prevents diet-induced hepatic steatosis. Mice lacking SCD1 are protected against diet- and genetically-induced hepatic steatosis.^{13,14,17,20,21} Likewise, dietary ω -3 PUFA supplementation protects against hepatic steatosis in a number of experimental models.^{6,22} It remains possible that the anti-steatotic actions of fish oil may be attributable in part to its ability to downregulate the hepatic expression of SCD1 (Supplemental Figure 1A,^{6,7}). As expected, in mice fed a SFA-rich diet, SCD1 ASO treatment alone resulted in striking reductions in hepatic neutral lipid accumulation (Supplemental Figure 4, A and B). In fact, in SFA-fed mice, SCD1 inhibition reduced hepatic TG by 93% (Supplemental Figure 3A) and hepatic cholesteryl ester mass (CE) by 81% (Supplemental Figure 3B), compared to control ASO treated mice. Likewise, dietary fish oil supplementation decreased hepatic TG by 93-96% on both diets (Supplemental Figure 3A). After 20 weeks of treatment, the rank order for the groups in regards to hepatic TG levels was: SFA-fed/Control ASO (1409 mg/g protein) > Fish-fed/Control ASO (462 mg/g protein) > SFA-fed/SCD1 ASO (96 mg/g protein) > Fish-fed/SCD1 ASO (19 mg/g protein). In contrast to neutral lipids, hepatic phospholipid mass was not different among the four groups (Supplemental Figure 3C). As expected³, SCD1 ASO treatment caused a striking decrease in the oleate (18:1) to stearate (18:0) ratio in hepatic TG in both diet groups (Figure 3D). Dietary fish oil alone also caused a significant reduction in 18:1 to 18:0 ratio, compared to the dietary SFA group (Supplemental Figure 3D), in agreement with the previously described fish oil-driven reductions in hepatic SCD1 activity (Supplemental Figure 1C). In addition, dietary fish oil supplementation resulted in expected ω -3 PUFA-enrichment in hepatic TG (Supplemental Figure 3F). Interestingly, ASO-mediated inhibition of SCD1 in mice fed a

fish oil diet caused significantly more ω -3 PUFAs to be incorporated into hepatic TG, compared to control ASO treated mice. The reduction in hepatic TG seen with both dietary fish oil and SCD1 inhibition is likely due in part to both of these treatments causing robust downregulation of genes involved in *de novo* fatty acid synthesis.^{6,9} In support of this, both dietary fish oil and SCD1 ASO treatment caused dramatic decreases in lipogenic genes including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and mitochondrial glycerol-3-phosphate acyltransferase (mGPAT) to a similar degree (Supplemental Figure 3E). Unlike genetic deletion of SCD1 in mice fed a very low fat diet,²³ SCD1 ASO treatment did not alter the expression of lecithin:cholesterol acyltransferase (LCAT) expression in mice fed either diet (Supplemental Figure 3E). Interestingly, both dietary fish oil and SCD1 ASO treatment caused an 81-86% decrease in hepatic expression of 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase) (Supplemental Figure 3E). As previously described³, SCD1 ASO treatment results in a ~2.2-fold increase in hepatic cholesterol 7 α -hydroxylase (Cyp7 α) expression, regardless of diet (Supplemental Figure 3E). Collectively, these data support previous observations that demonstrate both dietary fish oil and SCD1 inhibition are efficacious in the prevention hepatic steatosis.^{6,13,14,17,20,21}

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Supplemental Table I.

Fatty acid composition of diets (% of total FA)

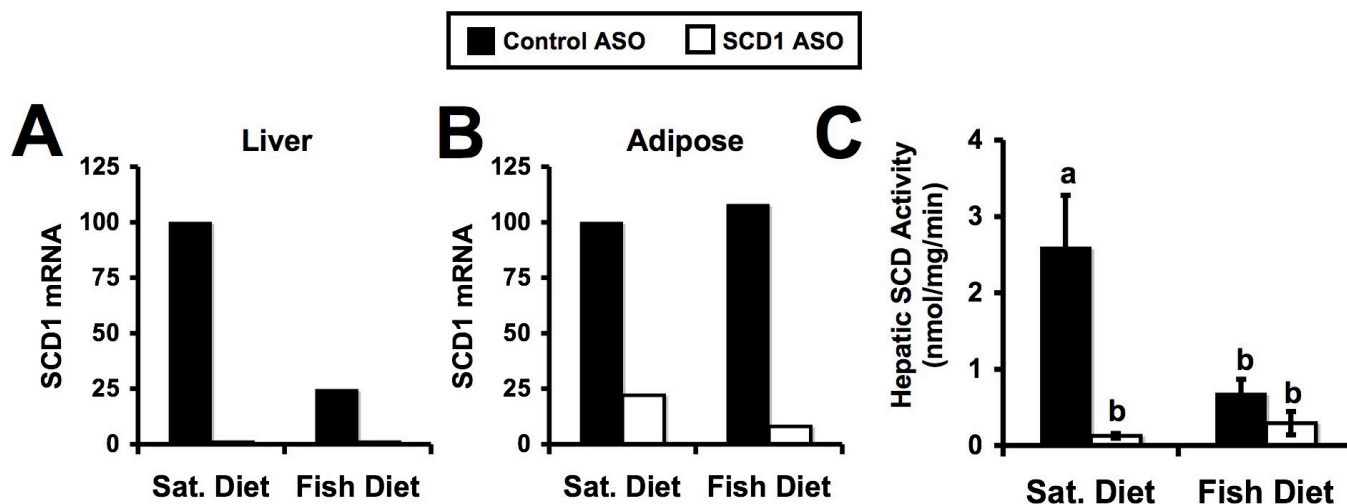
	12:0	14:0	16:0	16:1	18:0	18:1 (n-9)	18:2 (n-6)	18:3 (n-3)	20:0	20:4 (n-6)	20:5 (n-3)	22:6 (n-3)
Saturated Diet	2.0	6.4	31.7	0.7	6.1	24.4	17.9	0.5	0.2	0.5	0.5	0.5
Fish Oil Diet	0.1	6.1	20.4	10.3	4.5	13.5	6.4	0.3	0.1	0.9	10.4	8.5

Saturated Diet

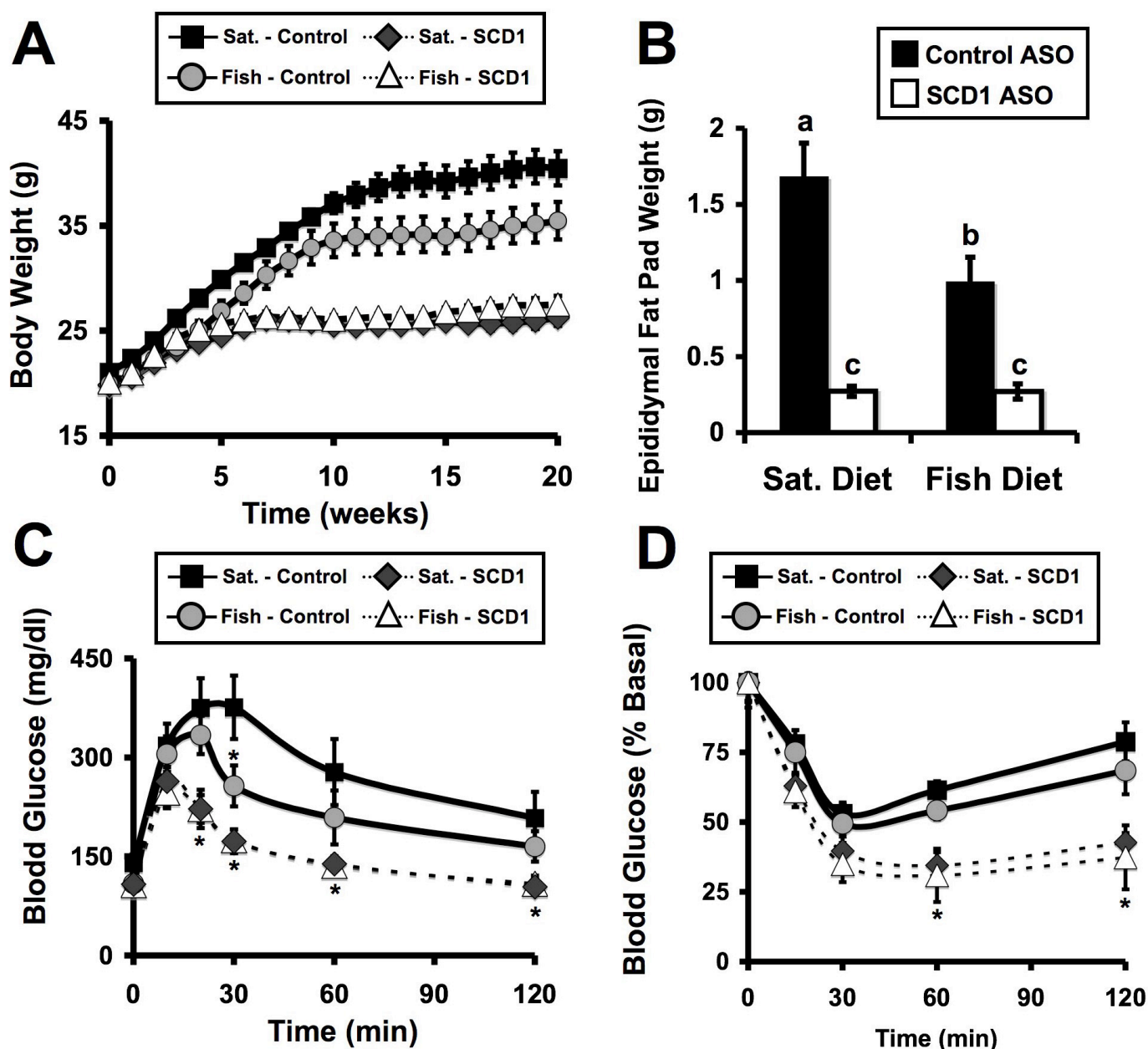
	g / 100 g	Fat (g)	Carbohydrate (g)	Protein (g)	Cholesterol (mg)	Phytosterol (mg)	α -tocopherol (mg)
Sat. Blend (AC Humko)	4.00	4.00				1.60	0.76
Fish Oil (Omega Protein)	0.22	0.22			1.09		0.02
Casein	8.00			8.00			
Lactalbumin	4.00			4.00			
Dextrin	17.00		17.00				
Sucrose	17.00		17.00				
Wheat Flour, Self-Rising	35.00	0.35	25.90	3.15		1.93	0.53
Alphacel (Fiber)	7.13						
Crystalline Cholesterol	0.10				100.00		
Vitamin Mixture, Teklad	2.50		1.03				30.28
Hegsted Salt Mixture	5.00						
β -sitosterol	0.03					32.00	
Tenox 20A (antioxidant)	0.004						
Vitamin E 5-67	0.002						1.34
TOTAL	100	4.6	60.9	15.2	101.1	35.5	33.7
Kcal		40.4	243.7	60.6			
% of Energy		12	71	17			

Fish Oil Diet

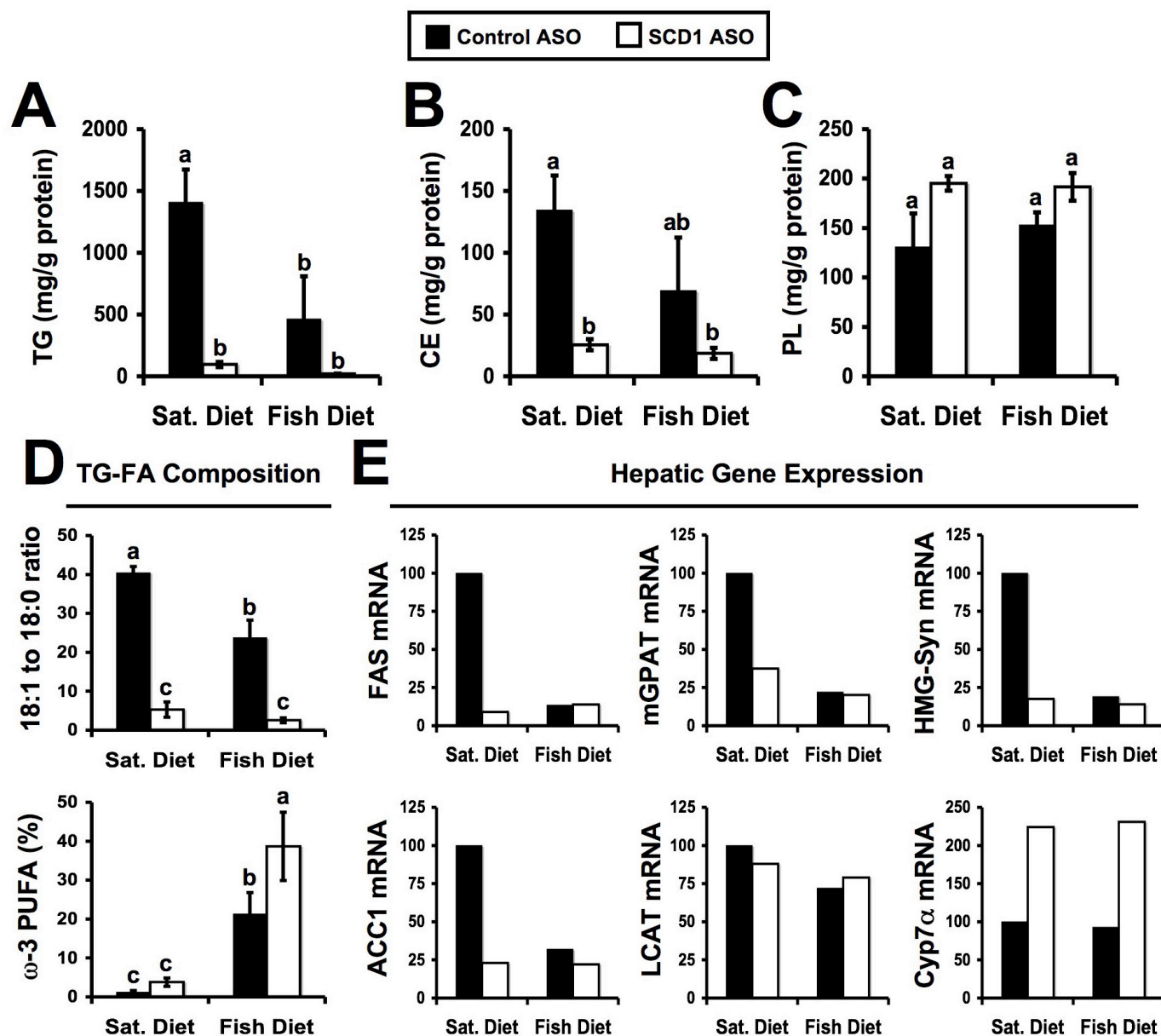
	g / 100 g	Fat (g)	Carbohydrate (g)	Protein (g)	Cholesterol (mg)	Phytosterol (mg)	α -tocopherol (mg)
Sat. Blend (AC Humko)	0.00					1.60	0.76
Fish Oil (Omega Protein)	4.20	4.20			20.83		0.02
Casein	8.00			8.00			
Lactalbumin	4.00			4.00			
Dextrin	17.00		17.00				
Sucrose	17.00		17.00				
Wheat Flour, Self-Rising	35.00	0.35	25.90	3.15		1.93	0.53
Alphacel (Fiber)	7.13						
Crystalline Cholesterol	0.08				80.00		
Vitamin Mixture, Teklad	2.50		1.03				30.28
Hegsted Salt Mixture	5.00						
β -sitosterol	0.03					32.00	
Tenox 20A (antioxidant)	0.004						
Vitamin E 5-67	0.002						1.34
TOTAL	100	4.6	60.9	15.2	101.1	35.5	33.7
Kcal		40.4	243.7	60.6			
% of Energy		12	71	17			



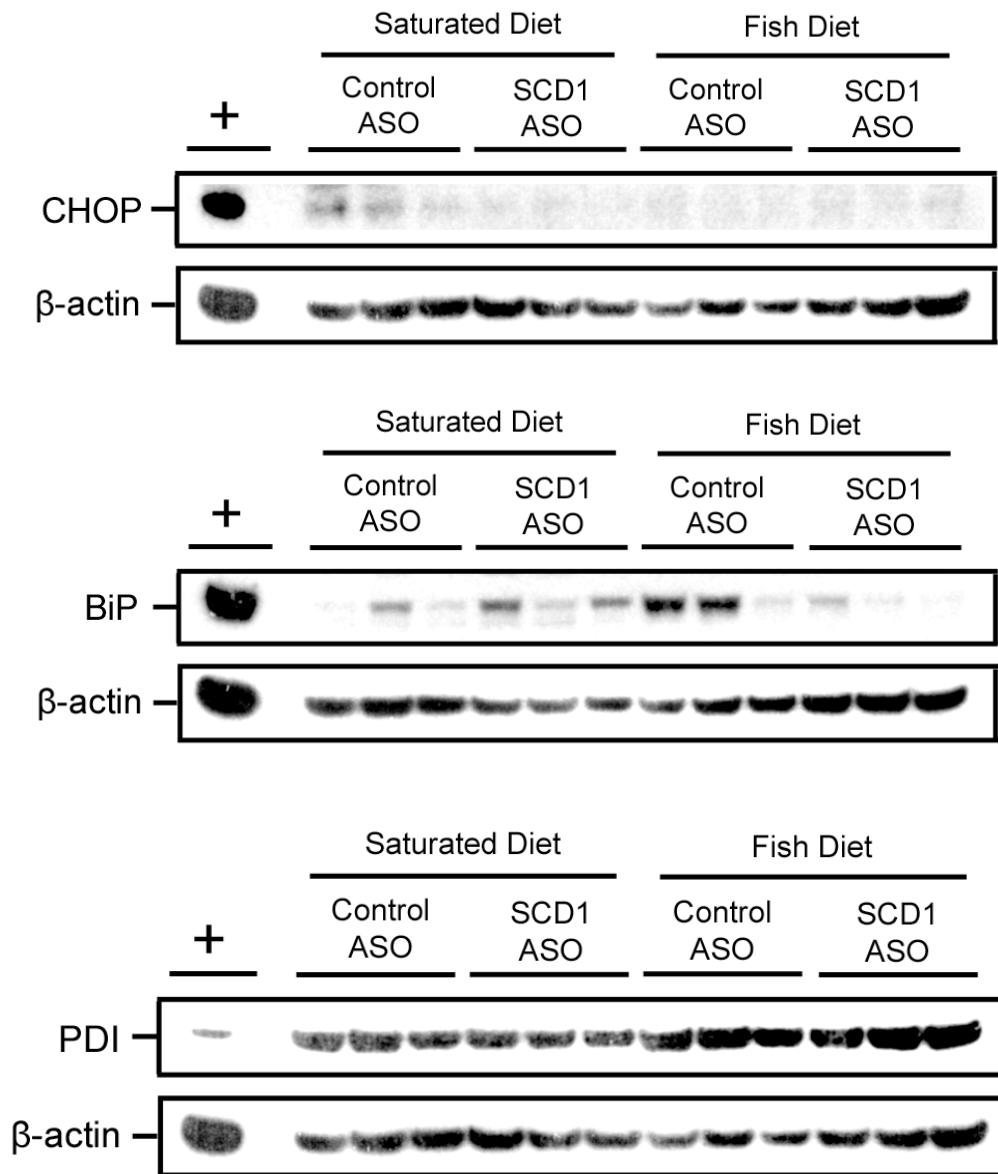
Supplemental Figure 1. Dietary and ASO-mediated regulation of SCD1 expression and function. Male LDLr^{-/-}, ApoB^{100/100} mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω -3 fatty acids (Fish) for 20 weeks in conjunction with biweekly injections (25 mg/kg) of a non-targeting control ASO ■ or SCD1 ASO □. Relative quantification of SCD1 mRNA levels in the liver (**A**) or epididymal adipose tissue (**B**) was conducted by real-time qPCR, and normalized to cyclophilin. Data shown in panels **A** and **B** represent pooled RNA samples with n=5 mice per group. **C.** Hepatic SCD1 activity, data represent the mean \pm SEM from 5 mice per group, and values not sharing a common superscript differ significantly ($p < 0.05$).



Supplemental Figure 2. Combination therapy of dietary fish oil and SCD1 ASO prevents diet-induced obesity and insulin resistance in $LDLr^{-/-} Apob^{100/100}$ mice. Starting at six weeks of age, mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω -3 fatty acids (Fish) for a period up to 20 weeks in conjunction with biweekly injections (25 mg/kg) of either a non-targeting control (control) or SCD1 ASO (SCD1). **(A)** Body weight, and **(B)** epididymal fat pad mass of mice following 20 weeks of diet and ASO treatment. Data in panel **B** represent the mean \pm SEM from 8-15 mice per group, and values not sharing a common superscript differ significantly ($p < 0.05$). Glucose tolerance tests **(C)** and insulin tolerance tests **(D)** were performed following 16 weeks of diet and ASO treatment. Data shown in panels **C** and **D** represent the mean \pm SEM from 5 mice per group, * = significantly different than the Sat.-Control group within each time point ($p < 0.05$).



Supplemental Figure 3. Combined therapy of dietary fish oil and SCD1 ASO prevents diet-induced hepatic steatosis in $LDLr^{-/-}$, $ApoB^{100/100}$ mice. Starting at six weeks of age, male mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω -3 fatty acids (Fish) for 20 weeks in conjunction with biweekly injections (25 mg/kg) of a non-targeting control ASO (Control) or SCD1 ASO (SCD1). Hepatic lipid mass measurements were conducted for **A.** triglyceride (TG), **B.** cholesteryl ester (CE), and **C.** phospholipids (PL). Data shown in panels **A-C** represent the mean \pm SEM from 6-8 mice per group; values not sharing a common superscript differ significantly ($p < 0.05$). **D.** Fatty acid (FA) composition [18:1 to 18:0 ratio or % of total FA as long chain ω -3 (eicosapentaenoic and docosahexaenoic) fatty acids] of hepatic triglycerides (TG-FA). Data shown in panel **D** represents the mean \pm SEM from 6-8 mice per group, and values not sharing a common superscript differ significantly ($p < 0.05$). **(E)** Hepatic gene expression was measured in pooled samples ($n=5$ per pool) by qPCR, and normalized to cyclophilin. The mRNAs examined include: fatty acid synthase (FAS), acetyl-CoA carboxylase 1 (ACC1), mitochondrial glycerol-3-phosphate acyltransferase-1 (mGPAT), lecithin:cholesterol acyltransferase (LCAT), 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-Syn), cholesterol 7 α -hydroxylase (Cyp7 α).



Supplemental Figure 4. Hepatic endoplasmic reticulum (ER) stress is not altered by diet or ASO treatment. Starting at six weeks of age, male mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω -3 fatty acids (Fish) for 20 weeks in conjunction with biweekly injections (25 mg/kg) of a non-targeting control ASO (Control) or SCD1 ASO (SCD1). ER stress was quantified by immunoblotting as described in materials and methods. The positive control (+) for this experiment represents 25 μ g of protein lysate from tunicamycin treated (10 μ g/ml for 10 hours) elicited peritoneal macrophages, which exhibit marked ER stress.

Supplemental Table II. Plasma cytokine levels following 20 weeks of diet and ASO treatment. Data represents the mean \pm SEM (n = 5); N.D. = values not detectable. Values not sharing a common superscript differ significantly (p < 0.05).

Plasma Cytokine Levels (pg/ml)				
	Sat. - Control	Sat. - SCD1	Fish - Control	Fish - SCD1
IL-1β	30.5 \pm 18.8 ^a	8.3 \pm 3.5 ^a	N.D.	6.6 \pm 4.3 ^a
IL-6	12.9 \pm 4.8 ^a	5.4 \pm 1.6 ^{ab}	7.0 \pm 2.5 ^{ab}	0.9 \pm 0.9 ^b
IL-12-p40	411.4 \pm 39.7 ^{ab}	448.7 \pm 34.3 ^a	246.2 \pm 31.2 ^c	343.3 \pm 25.0 ^{bc}
KC	205.3 \pm 67.0 ^a	142.8 \pm 16.3 ^a	132.9 \pm 28.9 ^a	107.6 \pm 11.0 ^a
MCP-1	101.9 \pm 7.5 ^a	100.9 \pm 14.6 ^a	65.9 \pm 5.5 ^b	90.5 \pm 14.8 ^{ab}
MIP-1α	10.5 \pm 10.5 ^a	2.4 \pm 2.4 ^a	N.D.	5.0 \pm 5.0 ^a
TNFα	10.5 \pm 10.5 ^a	1.2 \pm 1.2 ^a	N.D.	2.5 \pm 2.5 ^a
RANTES	12.6 \pm 1.3 ^{ab}	18.2 \pm 5.2 ^a	5.4 \pm 1.1 ^b	21.8 \pm 4.8 ^a